JC07 Rec'd PCT/PTO 05 MAR 2002

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **294-123 PCT/US**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5): **10/070523**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL00/00611	**September 1, 2000**	**September 2, 1999**

TITLE OF INVENTION

IMPROVED METHODS AND MEANS FOR RETROVIRAL GENE DELIVERY

APPLICANT(S) FOR DO/EO/US

Monique Maria Andrea Verstegen, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070523	PCT/NL00/00611	294-123 PCT/US

24. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	22 - 20 =	2	x $18.00	**$36.00**	
Independent claims	2 - 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims (**check if applicable**).			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$926.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$926.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$926.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (**check if applicable**). ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$926.00**	
				Amount to be: refunded	**$**
				charged	**$**

a. ☒ A check in the amount of **$926.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **08-2461** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Roanld J. Baron
NAME

29,281
REGISTRATION NUMBER

March 4, 2002
DATE

10/070523
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	Verstegen, Monique, Maria, Andrea, et al.	Examiner:	Unassigned
Serial No:	Unassigned	Group Art Unit:	Unassigned
International Appl. No.	PCT/NL00/00611	Docket:	294-123 PCT/US
Filed:	Herewith	Dated:	March 4, 2002
For:	IMPROVED METHODS AND MEANS FOR RETROVIRAL GENE DELIVERY		

Assistant Commissioner for Patents
BOX PCT
Washington, DC 20231
Attn: DO/EO/US

PRELIMINARY AMENDMENT

Sir:

In order to place the present application in condition for examination on the merits Applicant submits the following amendment for entry in the above-identified application.

IN THE SPECIFICATION:

On page 1, before line 1, after the title, please insert the following:

This application is the U.S. National Phase of International Application Number PCT/NL00/00611, filed September 1, 2000, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

On page 2, between lines 22 and 23 insert the following:

SUMMARY OF THE INVENTION

IN THE CLAIMS:

Please amend Claims 3, 5, 6, 10 and 14-20 to read as follows:

3. (Amended) A method according to claim 1 wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof.

5. (Amended) A method according to claim 1, wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells.

6. (Amended) A method according to claim 1 wherein composition of retroviral gene delivery vehicles of improved titer is applied.

10. (Amended) A method according to claim 8 wherein said producer cells are cells of hematopoietic origin.

14. (Amended) A composition according to claim 12 wherein said retroviral particles are capable of transducing hematopoietic stem cells and/or progenitor cells.

15. (Amended) A composition according to claim 12 wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

16. (Amended) A composition according to claim 12 for use as a pharmaceutical.

17. (Amended) Use of a composition according to claim 12 in the transduction of CD34 positive target cells.

18. (Amended) A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition according to claim 12.

19. (Amended) A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells, said composition being obtainable by a method according to claim 1.

20. (Amended) Use of a composition according to claim 18 in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

Please add the following new claims:

21. (Amended) A method according to claim 7 wherein said producer cells are cells of hematopoietic origin.

22. (Amended) Use of a composition according to claim 19 in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

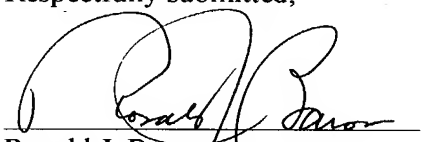
REMARKS

In order to place the present application in condition for examination in the U.S. Patent Office, Applicant has amended the Specification and Claims to conform to U.S. practice. No new subject matter has been introduced as a result of this Amendment. As a result of the present Amendment, Claims 1-22 remain in the application for purpose of prosecution.

The present Amendment has eliminated multiple dependent claims. Therefore, no additional fees should be assessed as a result of filing multiple dependent claims.

Since new matter has not been introduced as a result of this Amendment, entry hereof and examination and favorable consideration are respectfully requested. Any questions regarding this matter should be directed to the undersigned.

Respectfully submitted,



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VERSION OF AMENDMENT WITH MARKS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

On page 1, before line 1, after the title, please insert the following:

This application is the U.S. National Phase of International Application Number PCT/NL00/00611, filed September 1, 2000, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

On page 2, between lines 22 and 23 insert the following:

SUMMARY OF THE INVENTION

IN THE CLAIMS:

Please amend Claims 3, 5, 6, 10 and 14-20 to read as follows:

3. (Amended) A method according to claim 1 [or 2] wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof.

5. (Amended) A method according to claim 1 [any one of claims 1-4], wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells.

6. (Amended) A method according to claim 1 [any one of the afore going claims] wherein composition of retroviral gene delivery vehicles of improved titer is applied.

10. (Amended) A method according to claim 8 [any one of claims 7-9] wherein said producer cells are cells of hematopoietic origin.

14. (Amended) A composition according to claim 12 [or 13] wherein said retroviral particles are capable of transducing hematopoietic stem cells and/or progenitor cells.

15. (Amended) A composition according to claim 12 [any one of claims 12-14] wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

16. (Amended) A composition according to claim 12 [any one of claims 12-15] for use as a pharmaceutical.

17. (Amended) Use of a composition according to claim 12 [any one of claims 12-15] in the transduction of CD34 positive target cells.

18. (Amended) A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition according to claim 12 [any one of claims 12-15].

19. (Amended) A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells, said composition being obtainable by a method according to claim 1 [any one of claims 1-11].

20. (Amended) Use of a composition according to claim 18 [or 19] in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

Please add the following new claims:

21. (Amended) A method according to claim 7 [any one of claims 7-9] wherein said producer cells are cells of hematopoietic origin.

22. (Amended) Use of a composition according to claim [18 or] 19 in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

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Title: Improved methods and means for retroviral gene delivery

The present invention relates to the field of recombinant retroviral particles, to retroviral gene delivery vehicles. To methods for producing particles and vehicles as well as uses of the particles or vehicles in improved transduction methods, compositions for transduction as well as pharmaceutical compositions for the treatment of disorders with a genetic component. In particular the invention provides in one of its embodiments a method of gene transfer into e.g. pluripotent hematopoietic stem cells and their descendants, enabling successful transduction of 90% of CD34+ cells, including transplantable cell populations comprising hematopoietic stem cells that give rise to progeny expressing the transduced gene(s). The use of the method e.g. includes and is included in a method for treatment of a variety of hereditary and acquired human diseases by transfer of therapeutically active genes into hematopoietic stem cells and genetic marking of suchs cells.

The hematopoietic system produces perpetually large numbers of blood cells, which have a limited life span and need to be perpetually renewed throughout the life of a mammal. This renewal is maintained through proliferation and differentiation of a small number of hematopoietic stem cells in the bone marrow. The definition of stem cells is not always clear within the art. Herein a functional definition is used, which defines stem cells as those cells capable of (long term) reconstitution of a hematopoietic system. This definition is often felt to include at least some early progenitor cells. Since blood cells virtually reach every organ, hematopoietic stem cells are a highly suitable target for gene therapy for a variety of hereditary and acquired diseases within and outside the hematopoietic system. Unfortunately, retrovirus mediated gene transfer has met with only limited success due to the difficulty of obtaining sufficient numbers of successfully transduced,

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transplantable, long-term repopulating hematopoietic stem cells.

Recent advances in understanding stem cell biology include the discovery of heterogeneity of stem cells both in terms of maturity as well as the discovery of novel growth factors thought to control their proliferation and differentiation and the possibility to purify hematopoietic stem cells by selection for the CD34 surface marker. Retrovirus mediated gene transfer has been greatly benefited by co-localization of cells and virus using a recombinant fibronectin fragment, whereas the importance of selecting a suitable retrovirus receptor has been recognized. Based on these advances, we have analyzed the variables influencing gene transfer during the transduction procedure and selected a highly efficient producer cell subclone. With our invention we have made the observation that several binding steps involving receptors and ligands on virus, cells and mediating adherence molecules are needed for successful transduction, each with their specific affinities. Hence, our invention discloses the finding that the transduction procedure should be highly dependent on the relative concentration of these molecules during the tranduction procedure.

Thus the invention provides a method for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

As stated herein before, the population of CD34 positive

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cells such as they can be found in e.g. umbilical cord blood or bone marrow includes the stem cells as defined herein above (i.e. the cells capable of long term repopulation). We have found that a very important variable in the efficiency of transduction is a ratio between number of cells (or cell concentration) and the number of transducing particles. According to our invention these should be optimized vis a vis one another, which may sometimes lead to increasing particle titers and/or target cell concentrations, but surprisingly also to decreasing viral particles and/or lowering cell concentrations. Therefore a range of concentrations should be tried with samples from the target population of cells. Gene delivery vehicles of retroviral origin are all vehicles comprising genetic material and/or proteinaceous material derived from retroviruses. Typically the most important features of such vehicles are the integration of their genetic material into the genome of a target cell and their capability to transduce stem cells. These elements are deemed essential in a functional manner, meaning that the sequences need not be identical to retroviral sequences as long as the essential functions are present. The methods of the invention are however especially suitable for recombinant retroviral particles, which have most if not all of the replication and reproduction features of a retrovirus, typically in combination with a producer cell having some complementing elements. Normally the retroviral particles making up the gene delivery vehicle are replication defective on their own. The invention is particularly suited for the production of gene delivery vehicles, however other retroviral particles can also be produced according to the invention.

In another embodiment according to the invention not only the concentration of target cells is optimized, but also the concentration of virus. As stated before, optimization of all concentrations involved in binding or interaction is preferred. In order to be able to modify virus titers high

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initial titers are preferred. Methods to arrive at those are also provided by the present invention. Thus the invention further provides a method wherein target cell concentrations are optimized further comprising optimizing the concentration of said gene delivery vehicles for optimal transduction efficiency. It is of course that a gene delivery vehicle is intended to read on any vehicle capable of delivering genetic material to a target cell, whether the genetic material is actually a gene, an antisense molecule or a cosuppressive nucleic acid (encoding molecule), etc. Useful nucleic acids to be provided to target cells, e.g. stem cells are well known in the art and include such molecules as to replace inborn errors/deficiencies of the hematopoietic system, which may include hemoglobin genes and their regulatory elements for the thalassemia's and sickle cell anemia's and sequences to repair the various forms of severe combined immunodeficiency, such as caused by adenosine deaminase deficiency and that known as severe X linked immunodeficiency, or genes encoding enzymes for diseases known as lysosomal storage diseases, such as Hurler's, Hunter's, Krabbe's and in particular Gaucher's disease and metachromatic leukodystrophy, or by introducing sequences that confer resistance of the progeny of hematopoietic stem cells to infectious agents, such as HIV, as well as the introduction of suicide genes for cancer therapy and marker genes to track the progeny of transplanted normal and/or malignant hematopoietic stem cells. Another factor involved in binding and/or interaction is a matrix binding both virus and target cell exemplified herein by fibronectin and retronectin. The optimization of their concentration is also part of the present invention. Thus the invention also provides a method as described above wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof, preferably further comprising optimizing the concentration of said fibronectin, retronectin or said functional equivalent for

optimal transduction efficiency.

Typically the target cells of the present invention comprise populations of CD34 positive cells, which are efficiently transduced by retroviral particles, preferred are those populations wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells. As stated above it is preferred to use high viral titers to be able to optimize all relevant concentrations vis a vis all binding and/or interaction steps. Therefore the invention also provides a method as described above wherein a composition of retroviral gene delivery vehicles of improved titer is applied. According to the invention preferably a method for improving the virus titer is applied which involves improving the producer cell line. Thus the invention also provides a method wherein said virus titer is improved by providing a culture of producer cells of a retroviral gene delivery vehicle, subcloning said culture of producer cells, culturing the resulting subclones and selecting the clones producing the highest virus titers, possibly based on multiple copies of the provirus due to reinfection. Apparently a number of cells from established producer cell lines loose some of their ability to produce effective retroviral particles. Subcloning appears to be a way to select for those cells retaining that ability. Other ways of selecting for such cells are also included in the present invention.

Another factor promoting the transduction efficiency is prior cryopreservation of the (CD34+) cells prior to use in the transduction procedure. Typically, umbilical cord blood harvests are cryopreserved prior to use, whereas rhesus monkey cells are predominantly used immediately after procurement, ether or not following stimulation or mobilization of the CD34+ by administration of G-CSF to the animals. Cryopreservation resulted in less variability of the obtained transduction frequencies and, in general, a much higher level of transduction. The mechanisms involved are not clear and may be related to the use of DMSO, damage to the

cell membrane, more prominent availability of the GALV-receptor protein and/or upregulation of the receptor gene expression. Therefore, in one embodiment a harvest of stem cells is frozen, optionally stored, and thawed prior to performing a transduction method of the invention.

The method of improving virus titers can also be used apart from the improvement of transduction. Thus the invention also provides a method for producing retroviral particles at high titers, comprising providing a culture of producer cells producing retroviral particles, subcloning said culture of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers. Again the invention is preferably applied to gene delivery vehicle production. Thus the invention provides in yet another embodiment a method as just described wherein said retroviral particles are gene delivery vehicles. Typically producer cells are well known in the art. The preferred ones are mouse fibroblast cells, originating from PG13 which is pseudotyped with the gibbon ape leukemia virus (GALV). GALV-receptors (GLV-1 or Pit-1) are present on human hematopoietic cells.

The invention also includes compositions obtainable by the methods of the invention. Thus included are compositions comprising retroviral particles at high titer obtainable by a method as disclosed above, preferably those wherein said retroviral particles are gene delivery vehicles. Preferably such a composition comprises retroviral particles capable of transducing hematopoietic stem cells and/or progenitor cells, preferably wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

The invention also provides the pharmaceutical use of these compositions, particularly in the treatment of diseases having a genetic component, such as the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies,

the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer. Typically the use of a composition comprising retroviral particles will involve the transduction of CD34 positive target cells. Such transduced cells are typically made in vitro and are also part of the present invention. Thus the invention provides a composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition of retroviral particles according to the invention, or a composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells, said composition being obtainable by a method according to the invention.

Detailed description

The object of the present invention is to provide a generally applicable method for retrovirus mediated transfer of therapeutic and marker genes into pluripotent hematopoietic stem cells.

The invention includes the unexpected and surprising finding that transduction is dependent on the concentration of target cells during the transduction period and that selection of (high titer) subclones of a by itself effective producer cell line decreases this dependence on cell concentration. These two findings enable a reproducible, highly efficient method of gene transfer into hematopoietic stem cells, which maintain their transplantability and provide descendant containing the gene of interest and expressing this gene following transplantation in recipient

subjects. Proof of principle will be obtained using transplantation of successfully marked CD34+ cells into irradiated nonhuman primates. For human umbilical cord blood stem cells, we have obtained proof of principle by transplantation of human umbilical cord blood stem cells in immunodeficient NOD/SCID recipients. To test for transduction of transplantable human multilineage and/or pluripotent hemaopoietic stem cells, EGFP transduced CD34+ cells were transplanted into immunodeficient mice irradiated with 3.5 Gy (g-rays total body irradiation. The mice were sacrificed at day 35 to measure content of repopulating cells and to assess the multilineage nature of the transduced cells by flow cytometry. Typically, the optimized procedure resulted in percentages of up to 80% EGFP expression (Table 1) which was multilineage in nature (Figure 4). Since only a small subset of $CD34^+$ cells has the capacity to produce offspring after transplantation it was thought to be of interest to relate EGFP expression frequencies of $CD45^+$ cells in the engrafted NOD/SCID mice with the frequency of $EGFP^+$ cells in the transplanted $CD34^+$ cells (Figure 5). This analysis revealed a threshold of transduction of repopulating cells relative to the $CD34^+$ cells, which explains why substantial transduction frequencies in $CD34^+$ cells do not necessarily result in similar frequencies following transplantation, and also demonstrated that with the optimized procedure up to 80% of the multilineage repopulating cells should express the transgene.

For human umbilical cord blood stem cells, provisional proof of principle has already been obtained (Van Hennik et al., Blood, 1998) by transplantation of transduced stem cells into immunodeficient NOD/SCID recipients under conditions resulting in a lower transduction frequency of CD34+ than has become possible by the present invention. Hence, clinical application of the method for autologous umbilical cord blood gene therapy for a variety of diseases has become a feasible option for therapy by the present invention.

Materials and Methods

Viral vector and packaging cell line

The pseudotyped retroviral producer cell line PG13/EGFP7 was kindly provided by J. Barquinero (Institut de Recerca Oncologica, Barcelona, Spain). The cell line was developed by transducing the PG13 packaging cell line (kindly provided by D. Miller, Fred Hutchinson Cancer Research Centre, Seattle, WA) with 0,45 μm filtered supernatant from PA317/EGFP cell cultures. (Limon A et al., (1997), Blood, 90:3316-21 21). EGFP expression was analyzed by flow cytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flow cytometer coupled to an autoclone device (both from Coulter, Miami, FL, USA). Single clones were cultured as previously described. (Limon A et al., (1997), Blood, 90: 3316-21). The PG13/EGFP7 cell line was subcloned by diluting the cells to 1 cell per well of a 96-well plate. Single subclones were cultured as described and analyzed for transduction efficiency on rhesus BM en UCB CD34+ cells. The viral titer of the cell line (original and subclones) was in the order of 10^5 - 10^6 infectious particles per ml as determined by supernatant titration on cultured human HeLa cells and Rat-2 cells. Absence of replication-competent virus was verified by failure to transfer GFP-expression from a transduced cell population to a secondary population.

Subcloning of the PG13/SF-EGPP packaging cell line/vector combination (Figure 2)

Subcloning of the PG13/SF-EGFP7 virus producer cell line was performed using limiting dilution to grow one cell per well of a 96-well plate in culture medium consisting of an enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD). (Merchav S et al. (1984), Int J Cell Cloning, 2: 356-67). (Wagemaker G et al. (1980), Cell

Tissue Kinet, 13: 505-17). Supplemented with 10% FCS. Growing clones were harvested and grown in T75cm² flasks until 80% confluency and subsequently tested for transduction efficiency.

Determination of the virus titer (Figure 2)

To determine the virus titer of all clones, diluted supernatants of these clones were used to transduce cells of the Rat-2 cell line and HeLa cell line. The producer cell lines (PG13/SF-EGFP7, clone 1, clone 2, clone 3 and clone 5) were grown in T75 cm² culture flasks until 80% confluency as described above. Subsequently, 2000 Rat-2 cells and HeLa cells were cultured for 24 hours in dilutions of 0,45 μm filtered virus supernatant of the different clones of the virus producer cell line in 12 wells of a 24-wells plate. As a control, 1 well did not contain virus supernatant but culture medium solely. The virus supernatant was removed and substituted with fresh culture medium. The transduced cells were harvested at confluency and the transduction efficiency was determined by flow cytometry (FACSCalibur, Becton & Dickinson). The virus titer was determined by calculating the number of cells initially cultured (2000) that were transduced at a certain dilution of the virus supernatant.

Retroviral transduction

Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium consisting of serum-free enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD). (Merchav S et al. (1984), Int J Cell Cloning, 2: 356-67). (Wagemaker et al. (1980), Cell Tissue Kinet, 13: 505-17). Media for all cultures routinely included 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15' with read-outs between 9,5% and 10%) in a humidified atmosphere. The culture supernatant was

subsequently produced and passed through a 0,45 μm filter. To enhance the transduction efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 μg/cm² as described previously. (Moritz T et al. (1996), Blood, 88:855-62). CD34-selected UCB, human BM, normal rhBM or rhBM from G-CSF and/or Flt3-L treated monkeys were prestimulated for 2 days in enriched Dulbecco's medium with combinations of the human recombinant growth factors Flt3-L (50 ng/ml, kindly provided by Amgen, Thousand Oaks, CA, USA), thromopoietin (huTPO and rhTPO, 10 ng/ml, kindly provided by Genentech, South San Francisco, CA, USA) and stem cell factor (SCF, 100 ng/ml). Before adding purified subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the pseudotyped vector for 1 hour at 37°C. (Moritz et al. (1996), Blood, 88: 855-62). (Hanenberg H et al. (1996), Nat Med, 2: 876-82). Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with hematopoietic growth factors (HGF), and added to the dishes. Over a period of 2 days, culture supernatant was replaced completely by resuspending nonadherent cells into fresh retrovirus supernatant and HGF. Finally, the cells were harvested and analysed by flow cytometry and cell cycle analysis.

All umbilical cord blood samples used were cryopreserved prior to use, as were the indicated samples of rhesus monkey bone marrow. For cryopreservation, the cells were suspended in HEPES buffered Hanks' balanced salt solution, supplemented with 22,5% foetal calf serum and 7,5% DMSO in a concentration ranging from 20-200 x 10^6/ml. The cells were frozen in ampoules of 1, 1,5 or 5 ml volume using a Planer Biomed Kryo 10 controlled cryopreservation machine during the crystallization at a rate of -1°C per minute. Prior to use, cryopreserved cells were thawed by the standard so-called "step-wise dilution" method, thoroughly washed and

resuspended in the medium used for transduction.

Target cell titrations (Figure 1)

Rhesus monkey BM (rh BM) and human umbilical cord blood (UCB) cells were titrated from $3x10^6$ to 10^3/ml during the transduction assay. The producer cell line was, as standard, cultured in T75 cm^2 flasks filled with 10 ml serumfree medium as described above until 80% confluency. During the transduction, the virus supernatant was refreshed once. After 2 days prestimulation and 2 days of supernatant infection the cells were harvested and the transduction efficiency was determined by flow cytometry.

Transplantation of transduced human UCB cells into NOD/SCID mice

Male, specified pathogen-free (SPF) NOD/LtSz-scid/scid mice (NOD/SCID) were bred and housed under SPF conditions and supplied with sterile food and drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 μl H+H and injected i.v. into a lateral tail vein. Transplanted cell numbers were $2x10^5$ $CD34^+$ UCB cells. At day 35 after transplantation, the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs and the spleen were isolated and BM suspensions were prepared by flushing. After counting, BM cells were analyzed by flow cytometry to determine the percentage human $EGFP^+$ cells in the mouse BM and their multilineage nature determined by flow cytometry. Data were expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test.

Two tailed P values of <0.05, were considered significant. Actual significance levels are indicated in table 1 and in the figures.

Transplantation assays in rhesus monkeys

To test for transduction of transplantable multilineage and/or pluripotent hematopoietic stem cells, EGFP-transduced CD34+ cells are transplanted into rhesus monkeys subjected to 9 Gy (6 MV X-rays) total body irradiation in cell numbers range from hundred thousand to several millions of CD34+ cells per kg body weight, either immediately following transduction or selected for expression of the EGFP gene by cell sorting using a FACS Vantage cell sorter (Becton Dickinson). The transplantation procedure has been described in detail (Neelis KJ et al. (1997), Exp Hematol, 25:1094-103). The monkeys are followed daily for expression of EGFP in peripheral blood subsets, and weekly for expression in bone marrow subsets, using flow cytometric surface marker labeling to identify the different blood cell lineages. As indicated in figure 3 the transduction of stem cells using methods of the invention can be reproducibly very high.

Brief description of the drawings

Figure 1
Cell titration of CD34-selected rhesus BM cells (A) and human umbilical cord blood (UCB) cells. (B) during infection with the GALV-pseudotyped PG13/SF-EGFP7 retroviral packaging cell line/vector combination. The highest levels of EGFP$^+$ cells were found after transduction of $5x10^4$/ml rh BM cells or 10^5/ml UCB cells with 35% and 80%, respectively.

Figure 2
Subcloning of the PG13/SF-EGFP7 packaging cell line/vector combination resulted in clones with different virus titers as determined by supernatant dilution on Rat-2 cells (A) and HeLa cells (B). Clone 1 and 2 (PG13/SF-EGFP7.1 and PG13/SF-EGFP7-2) showed the highest virus titers, whereas clone 3 and 5 (PG13/SF-EGFP7.3 and PG13/SF-EGFP7.5) resulted in low virus titers. Transduction of $5x10^5$ rh BM cells with PG13/SF-EGFP7 and clones 1-5, 2b, 4b and 5b (C) resulted in levels of EGFP transduced rh BM cells that correlates with the virus titers. Clone 1, 2 and 2b showed EGFP levels of 80%-90% which was higher as compared to the parental PG13/SF-EGFP7 producer. Subclones of clone 1 and 5 (PG13/SF-EGFP7.1.2 and PG13/SF-EGFP7.5.1, respectively) resulted in similar transduction levels as the parental cell lines. Decreasing cell numbers (from 10^6 tot 10^3 cells/ml) during transduction using virus supernatant of PG13/SF-EGFP7 resulted in increasing transduction efficiency ranging from 10% to 40% (D). Transduction with supernatant from subclone PG13/SF-EGFP7.1 resulted in higher efficiencies (80% to 90% EGFP$^+$ cells) without the titration effect caused by increasing cell numbers/ml.

Figure 3
The effect of prior cryopreservation on transduction

efficiency. Briefly, rhesus monkeys were treated with 100 μg/kg G-CSF for 4 consecutive days, after which bone marrow was procured and used in the described transduction procedure after selection and isolation of cells expressing CD34. The figure shows the transduction frequencies obtained for bone marrow samples immediately used ("fresh") or cryopreserved ("frozen") in comparison to bone marrow from rhesus monkeys taken from a rhesus monkey bone marrow bank ("steady state rhBM"). The differences between the "frozen" and the "fresh" cells is statistically significant ($p=0.01$).

Figure 4
Chimerism and EGFP expression levels in a chimeric NOD/SCID mouse BM 35 days after transplantation of 10^5 $CD34^+$ UCB cells of which 93% expressed the EGFP gene. The bright green autofluorescence on the X-axes vs CD45 on the Y-axes clearly shows that almost all human ($CD45^+$) cells (80%) express the EGFP. The right panel shows the distribution of $EGFP^+$ (❑) and $EGFP^-$ cells (■) in all hematopoietic lineages assessed.

Figure 5
Percentage of EGFP positive $CD45^+$ cells in NOD/SCID mice 35 days after transplantation related to the percentage of primary EGFP positive $CD34^+$ cells transplanted. (●) AM12/MFG-EGFP transductions; (○) PG13/SF-EGFP transductions. The regression line of the data without the amphotropic transductions is identical to that shown of all data pooled.

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CLAIMS

1. A method for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

2. A method according to claim 1 further comprising optimizing the concentration of said gene delivery vehicles for optimal transduction efficiency.

3. A method according to claim 1 or 2 wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof.

4. A method according to claim 3 further comprising optimizing the concentration of said fibronectin, retronectin or said functional equivalent for optimal transduction efficiency.

5. A method according to any one of claims 1-4, wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells.

6. A method according to any one of the afore going claims wherein composition of retroviral gene delivery vehicles of improved titer is applied.

7. A method according to claim 6 wherein said virus titer is improved by providing a culture of producer cells of a retroviral gene delivery vehicle, subcloning said culture

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of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers.

8. A method for producing retroviral particles at high titers, comprising providing a culture of producer cells producing retroviral particles, subcloning said culture of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers.

9. A method according to claim 8, wherein said retroviral particles are gene delivery vehicles.

10. A method according to any one of claims 7-9 wherein said producer cells are cells of hematopoietic origin.

11. A method according to claim 10 wherein said producer cells originate from PG13.

12. A composition comprising retroviral particles at high titer obtainable by a method according to claim 8.

13. A composition according to claim 12 wherein said retroviral particles are gene delivery vehicles.

14. A composition according to claim 12 or 13 wherein said retroviral particles are capable of transducing hematopoietic stem cells and/or progenitor cells.

15. A composition according to any one of claims 12-14 wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

16. A composition according to any one of claims 12-15 for use as a pharmaceutical.

17. Use of a composition according to any one of claims 12-15 in the transduction of CD34 positive target cells.

18. A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition according to any one of claims 12-15.

19. A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of

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CD34 positive cells, said composition being obtainable by a method according to any one of claims 1-11.

20. Use of a composition according to claim 18 or 19 in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

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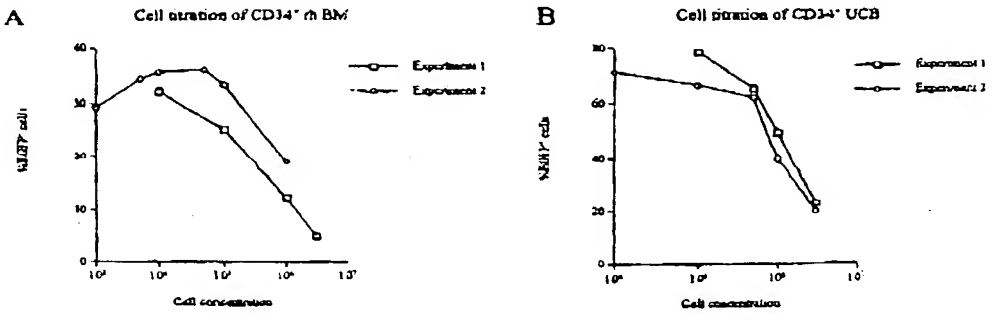

(57) Abstract: The invention provides methods, compositions and uses of said compositions for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

WO 01/16341 A1

Repopulation of EGFP-transduced $CD34^+$ UCB cells in NOD/SCID mice

Virus producer	Transduction efficiency %EGFP	$EGFP^+$ /chimeric mice	Chimerism in NOD/SCID %CD45	$\%EGFP^+$ in $CD45^+$ cells ± SD (range)
PG13/SF-EGFP7	66	4/4	8 (3-12)	23 ± 17 (2–41)
PG13/SF-EGFP7.1	85	5/5	3 (1-6)	63 ± 17 (38–80) *

* $p<0.009$

Table 1

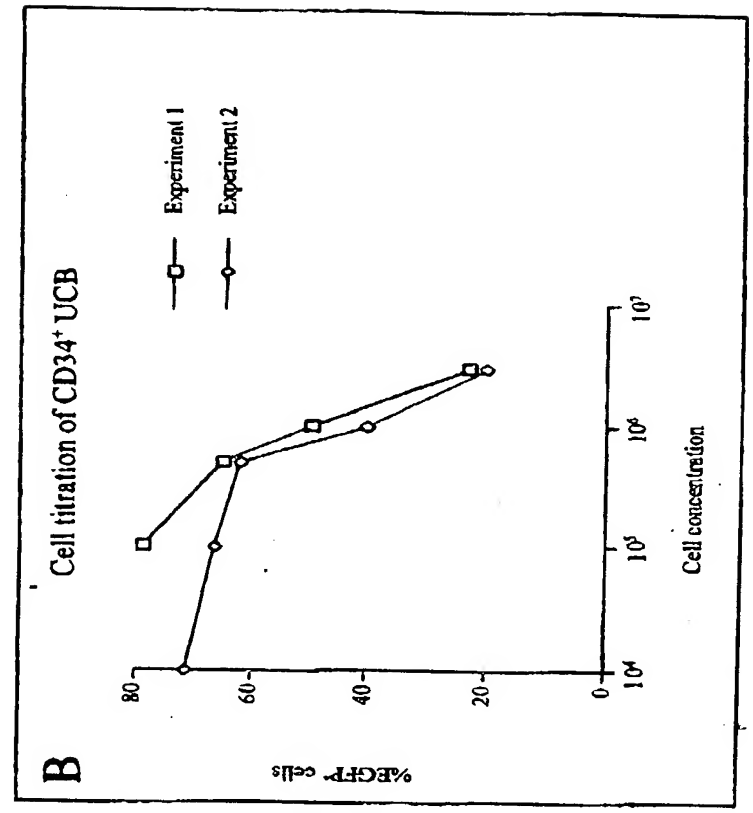
B
Cell titration of CD34+ UCB
Experiment 1
Experiment 2
%EGFP+ cells
Cell concentration
A
Cell titration of CD34+ rh BM
Experiment 1
Experiment 2
%EGFP+ cells
Cell concentration

Fig. 1

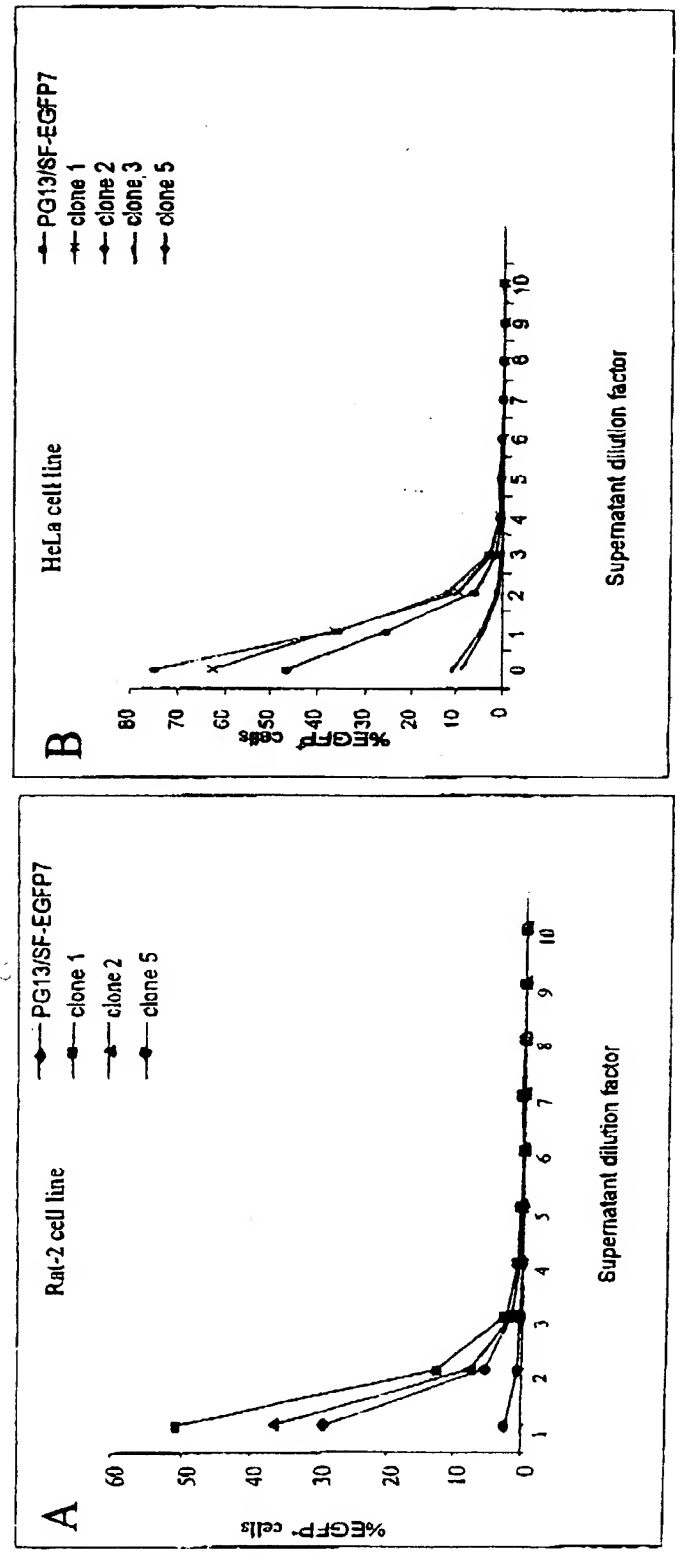
A
Rat-2 cell line
PG13/SF-EGFP7
clone 1
clone 2
clone 5
%EGFP+ cells
Supernatant dilution factor
B
HeLa cell line
PG13/SF-EGFP7
clone 1
clone 2
clone 3
clone 5
%EGFP+ cells
Supernatant dilution factor

Fig. 2

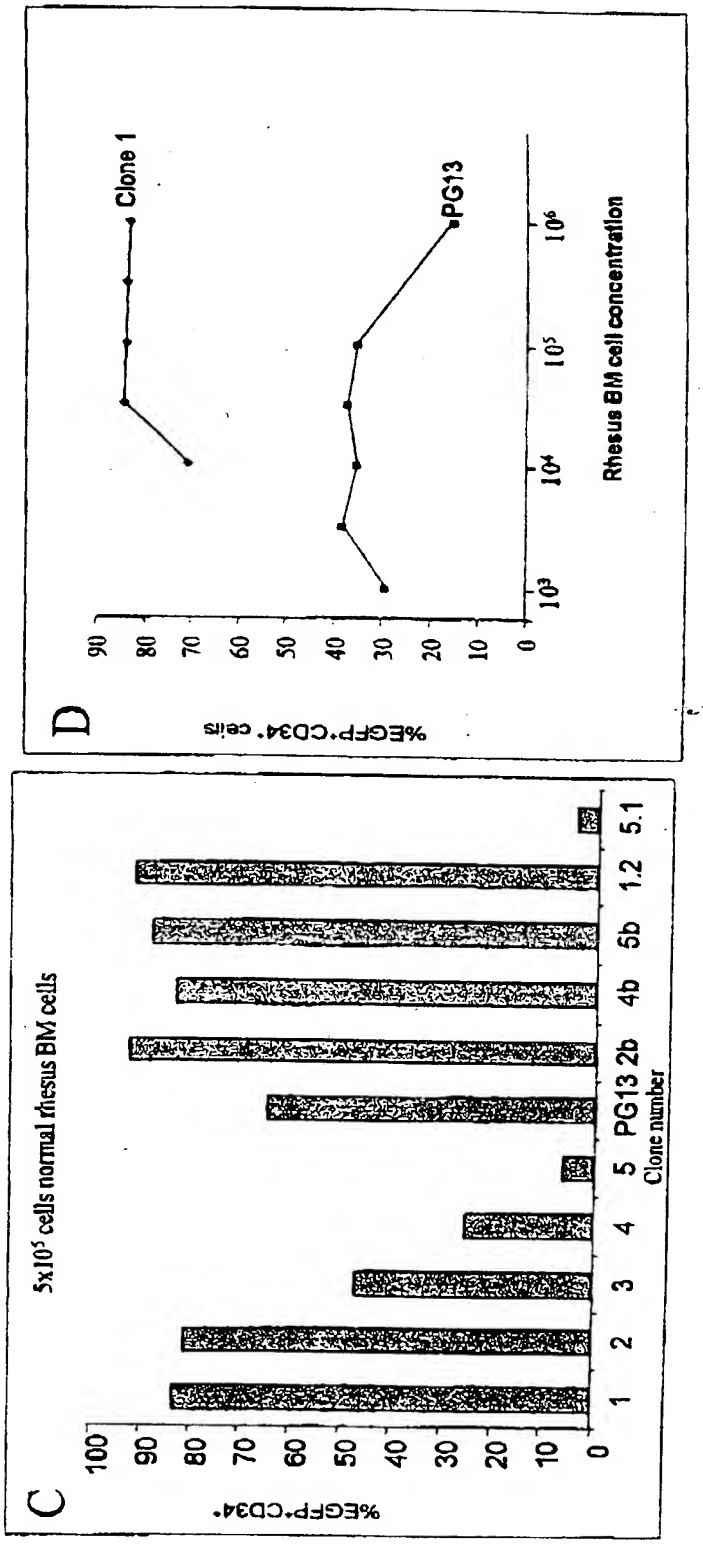
D
%EGFP+CD34+ cells
Clone 1
PG13
Rhesus BM cell concentration
C
5x10⁵ cells normal rhesus BM cells
%EGFP+CD34+
Clone number
1
2
3
4
5
PG13
2b
4b
5b
1.2
5.1

Fig. 2 (cont.)

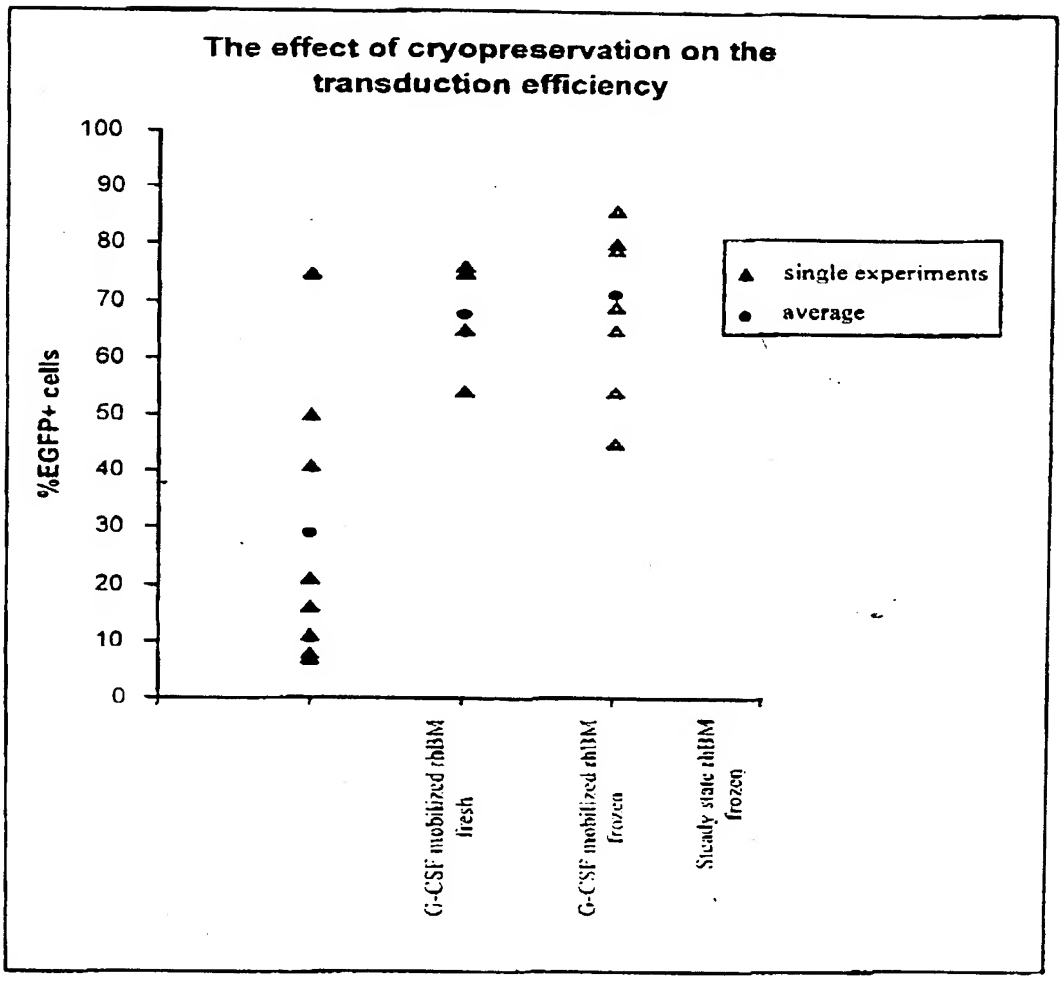
The effect of cryopreservation on the transduction efficiency
%EGFP+ cells
100
90
80
70
60
50
40
30
20
10
0
single experiments
average
G-CSF mobilized rhBM fresh
G-CSF mobilized rhBM frozen
Steady state rhBM frozen

Fig. 3

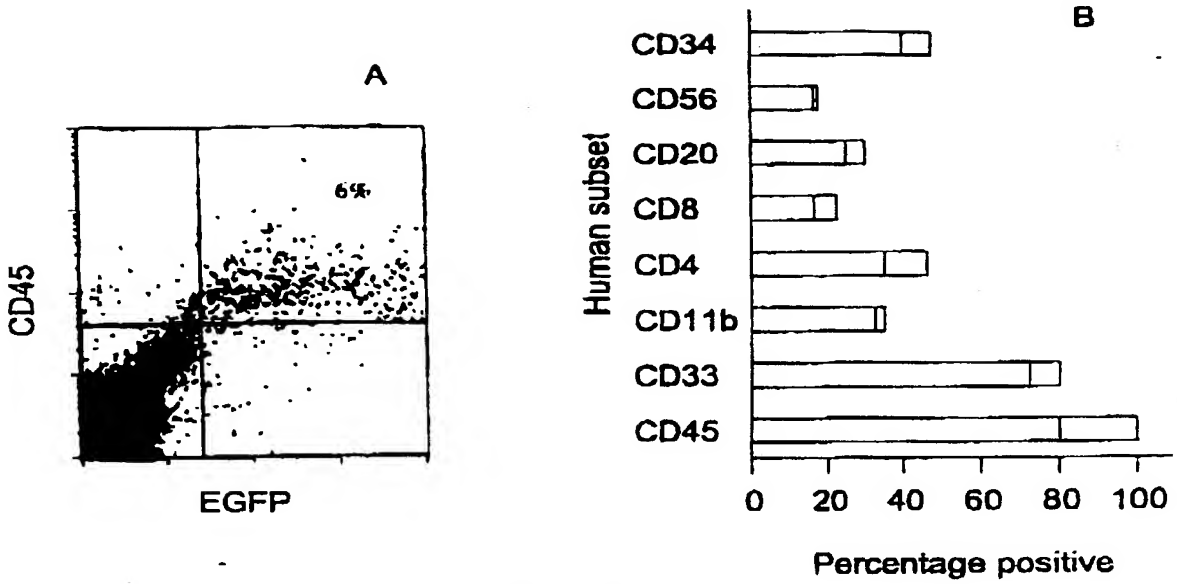
A
6%
CD45
EGFP
B
CD34
CD56
CD20
CD8
CD4
CD11b
CD33
CD45
Human subset
0
20
40
60
80
100
Percentage positive

Fig. 4

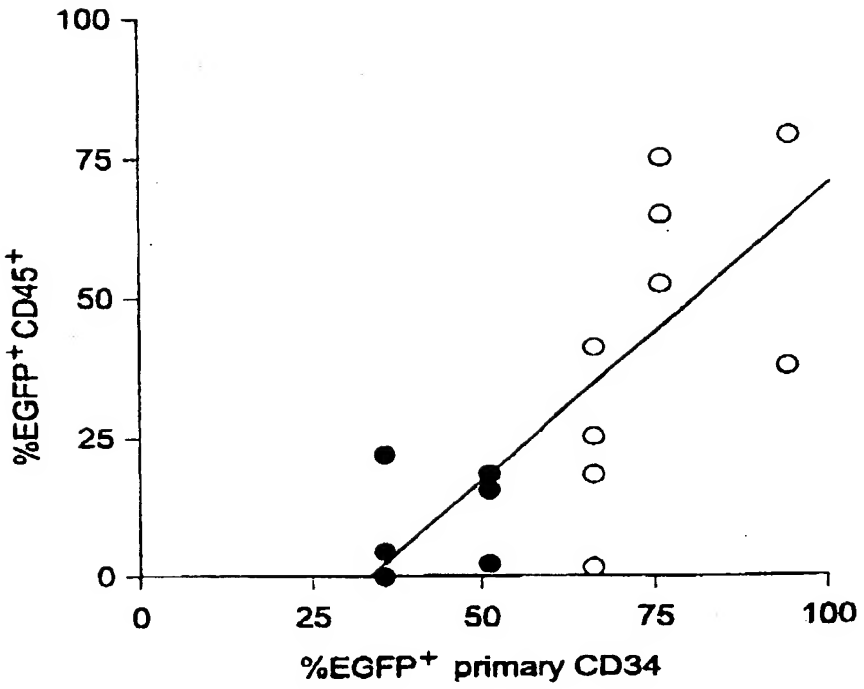
100
75
50
25
0
%EGFP+ CD45+
0
25
50
75
100
%EGFP+ primary CD34

Fig. 5

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(Design or Utility)

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My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: "Improved methods and means for retroviral gene delivery".

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Number 99202859.7	Country EP	Day/Month/Year Filed 2 September 1999
Number 99203875.2	Country EP	Day/Month/Year Filed 19 November 1999
Number	Country	Day/Month/Year Filed

Power of Attorney

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Signature of Inventor		Date

[redacted]

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Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date

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Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)

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(Design or Utility)

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: "Improved methods and means for retroviral gene delivery".

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Number	Country	Day/Month/Year Filed

Power of Attorney

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby authorize them or others whom they may appoint to act and rely on instructions from and communicate directly with the person/organization who/which first sends this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instructed otherwise.

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I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

Prior Provisional Application(s)	
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s), or under 35 U.S.C. §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R.§1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Prior U.S. or International Application(s)		
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C.§1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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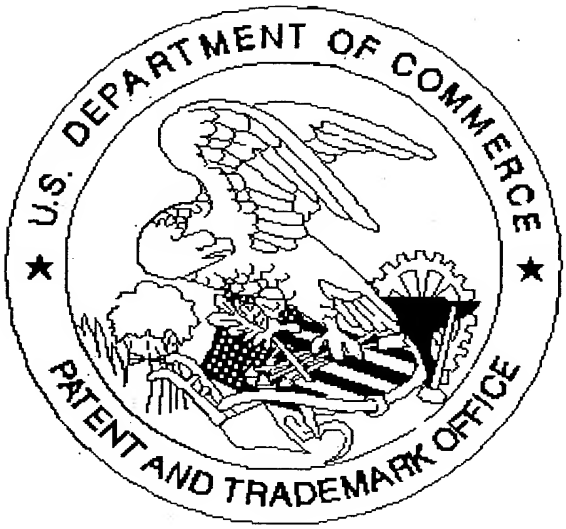

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